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14. ABSTRACT
Mammary gland samples were isolated from wild type, vitamin D receptor knockout (VDRKO) and 1alphahydroxylase knockout (1αKO) female mice for whole mounts and paraffin embedding (inguinal) and for RNA and protein isolation (thoracic). Time points collected included 6-10wk old nulliparous, 9 and 16 days pregnancy, 5 and 10 days lactation and 3 and 6 days involution. All whole mounts were completed and showed increased branching during pregnancy in the VDRKO glands relative to wild type and 1αKO glands. Paraffin embedded involution samples were stained with hematoxylin and eosin and showed an apparent decrease in alveolar breakdown in the first few days of involution in VDRKO and 1αKO glands compared to wild type controls. Organ culture studies show that treatment of wild type and 1αKO glands with 1,25 dihydroxyvitamin D retards tertiary branching. This appears not to be the case in VDRKO glands. Gene expression analysis via microarray and qPCR provides only a glimpse into the complexities of the signaling involved in this process and is being complimented by ongoing protein analysis. These results suggest that 1αKO mammary glands do not develop exclusively similar to VDRKO or wild type glands which verifies our need to complete the remainder of our studies to determine if the VDR is acting to control mammary gland development through the vitamin D pathway or through some other ligand or possibly without a ligand.

15. SUBJECT TERMS
Breast cancer, prevention, vitamin D, transgenic mice

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Introduction

Epidemiological studies have shown a significant increase in cancer incidence in women who are vitamin D deficient (1;2). It was also shown that there is a correlation between breast cancer death rate and distance from the equator, where lesser exposure of direct sunlight leads to lower endogenous vitamin D levels. These studies suggest that optimal vitamin D status may contribute to prevention of breast cancer in human populations. The processes of proliferation, differentiation and apoptosis of epithelial cells in the mammary gland are carefully regulated by the rise and fall of several hormones which control the budding and extension of ductal branching, alveolar development and regression of these structures at distinct developmental stages. Elevation of these signals occurs at puberty to cause the first major growth of the ductal network. Aberrant growth of mammary gland epithelial tissue may occur as a result of failure of cellular safeguards through familial or somatic mutation, which can lead to the onset of cancer. 1,25-dihydroxyvitamin D (1,25D) is a key regulator of calcium homeostasis but is also a cellular growth regulator in numerous tissues including prostate, skin, colon and breast (3). In the body, vitamin D is converted to 25-hydroxyvitamin D (25D) which is then activated by the 1-alphahydroxylase (1αOHase) enzyme to 1,25 D, which is the high affinity ligand for the transcriptional regulator vitamin D nuclear receptor (VDR) (4). The function of the VDR in calcium regulation has been studied extensively although many questions still remain as to its role in cellular proliferation, differentiation and apoptosis in individual tissues. Mechanisms of 1,25 D-VDR effects include inhibition of cyclin D1 (5;6), induction of cell cycle inhibitors p21 and p27 (7) and growth inhibitor TGF-β (8). 1,25 D promotes differentiation in breast cancer cells by upregulating β-casein (9;10). Also, induction of apoptosis has been observed in response to 1,25 D treatment in a number of cancer cell lines (including breast) due to generation of reactive oxygen species and subsequent release of the apoptosis-inducing cytochrome c (11). Downregulation of anti-apoptotic factor Bcl-2 and upregulation of pro-apoptotic factor Bax has also been observed in 1,25 D treated breast cancer cells *in vitro*. Few additional binding partners for the VDR have been established. β-catenin binds to the VDR while unliganded (without 1,25 D) (9). Prolonged activation of β-catenin can induce hair follicle tumors while loss of function of the VDR leads to alopecia (loss of hair) (12). This alopecia is not present in mice lacking 1αOHase enzyme suggesting it may be driven by promiscuous or unliganded VDR activity. The studies detailed here will help to determine how the vitamin D pathway affects growth within the mammary gland and whether the increased growth previously observed in mammary glands lacking the VDR is due to VDR specifically or the dysfunction of the vitamin D pathway. By using transgenic mouse models, these studies will provide valuable mammary gland contextual data that cell culture cannot provide.

Body

In vivo analysis of mammary gland development

(Subtask 1) Scheduling the pairings of our breeding colonies (to be completed by months 1-9) took longer than anticipated as we had the unexpected issue of lower than normal pup size in all of our colonies (figure 1). This was a concern since mammary gland development during puberty is linked to post-natal growth (13). After analysis of the growth of these pups, it was determined that we would use

the larger females at a later age for our study. Once this issue was addressed, the breeding and collection of samples went as anticipated and was finished at the beginning of month 12. We took mammary glands at the following time points: 4-10 week old nulliparous (puberty), 9 and 16 days pregnancy, 5 and 10 days post partum (lactation) and 3 and 6 days involution. This was done for all colonies (WT, VDRKO, 1αOHaseKO (1αKO)).

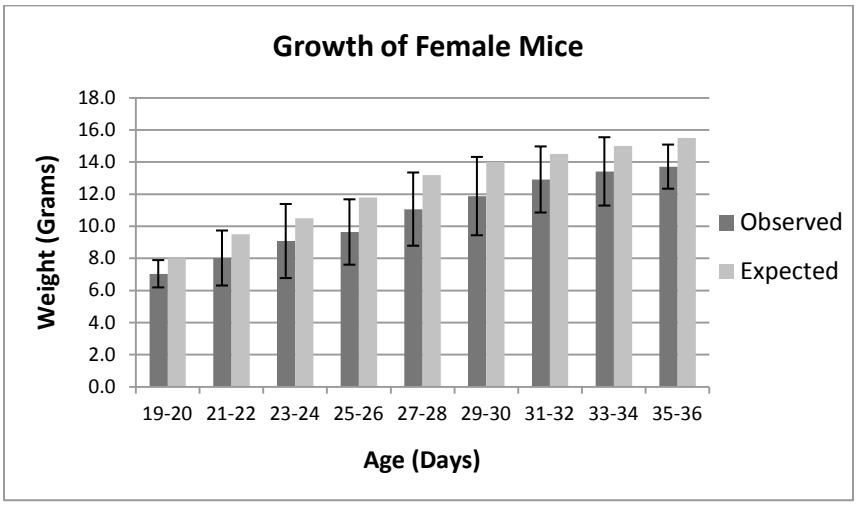

Figure 1. Weights of female weanlings were more variable and overall lower than anticipated.

(Subtask 2) Whole mounts were made from the right inguinal mammary glands of each of these mice (months 1-9), using a carmine alum stain. These stains were completed by the end of month 12. (Subtask 3) The ductal outgrowth of these glands was measured (months 3-5) and completed by the end of month 12 (figure 2). (Subtask 4) Terminal end bud formation was analyzed in pregnant mammary glands (months 4-6) and completed by the end of month 12 (figure 3).

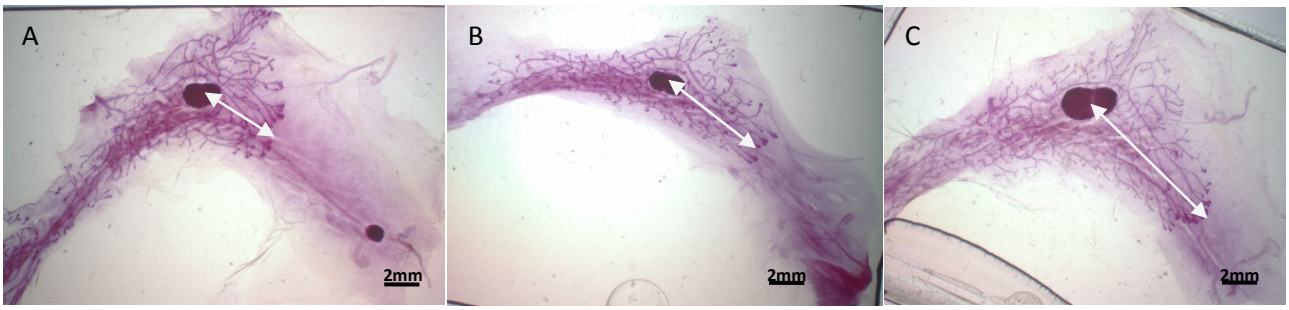

Figure 2. Whole mounts of mammary glands from 7wk old (A) WT, (B) 1αKO, (C) VDRKO female mice. Ductal outgrowth in VDRKO mice were shown to be higher than in WT controls.

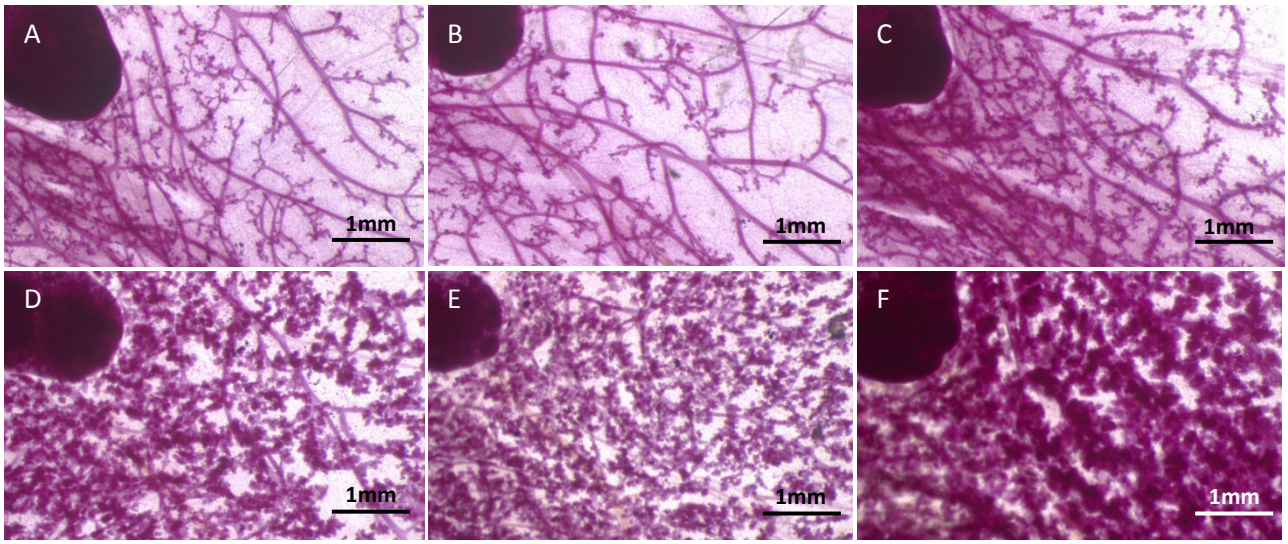

Figure 3. Whole mounts of mammary glands from 9 days (top) and 16 days (bottom) pregnant (A,D) WT, (B,E) 1αKO, (C,F) VDRKO female mice. Tertiary branching is more extensive in the VDRKO glands compared to the WT and 1αKO glands.

(Subtask 5) All left inguinal glands from each mouse were fixed in a 4% formalin solution overnight, processed in our tissue processor and embedded in blocks of paraffin wax (months 1-9). This was completed by the end of month 12. All blocks were sectioned at 5 microns and stained with hematoxylin and eosin (H&E) (figure 4). (Subtask 6) H&E stained sections for pregnancy and involution samples were analyzed for epithelial cell density (months 6-10) and was done so by the end of month 14 (figure 5).

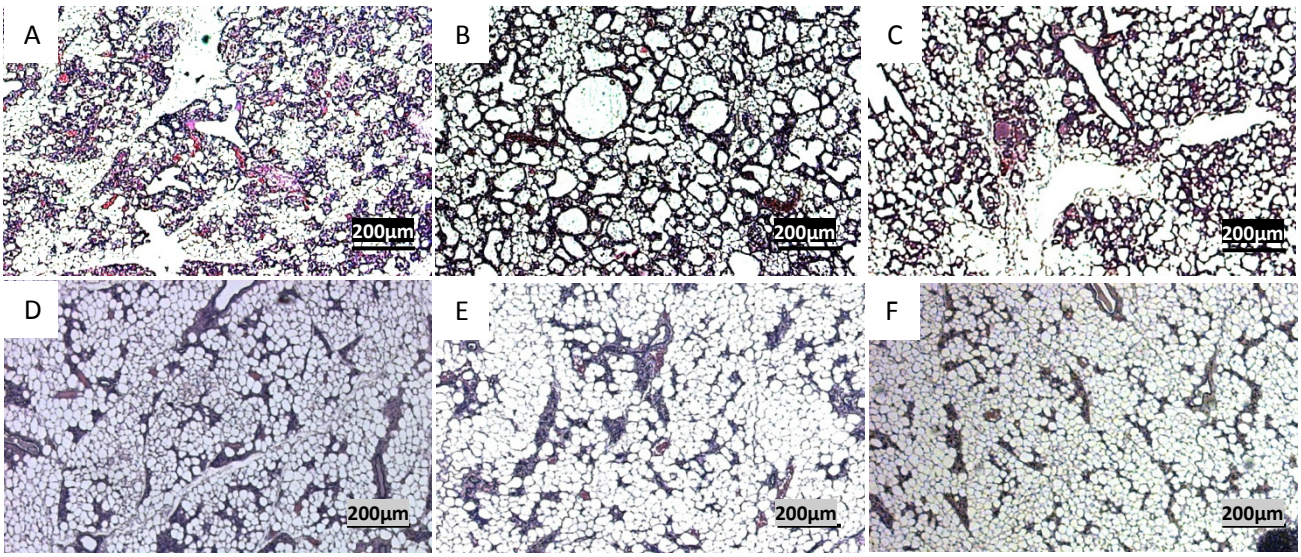

Figure 4. Hematoxylin and eosin (H&E) stained sections from (A,D) WT, (B,E) 1αKO, (C,F) VDRKO glands at 3 days involution (top) and 6 days involution (bottom). VDRKO and 1αKO mammary glands at 3 days involution consist of persistent larger alveoli while their wild type counterparts present much smaller alveoli, suggesting decreased sensitivity to post weaning signaling in those glands. This difference does not appear to be present in glands 6 days post weaning.

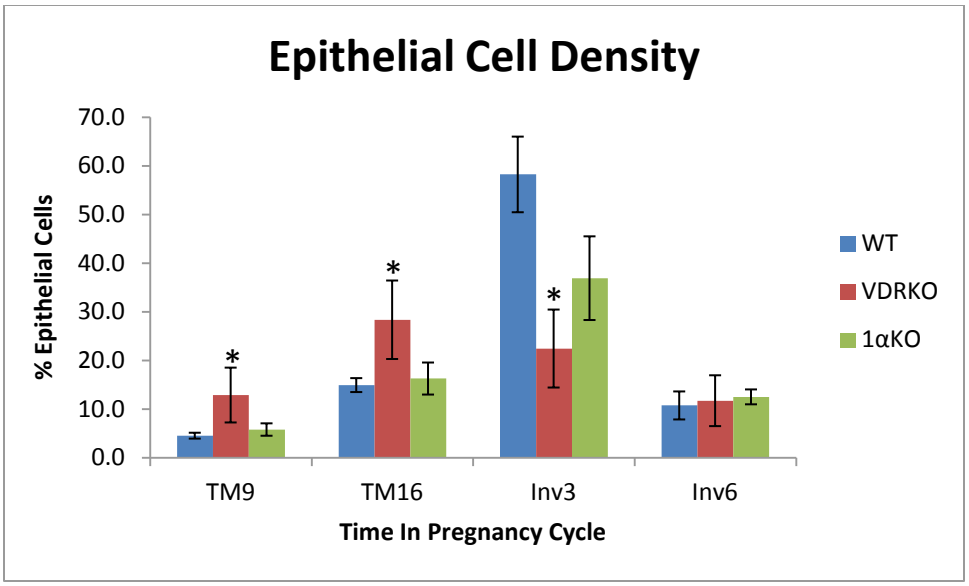

Figure 5. Quantification of hematoxylin and eosin (H&E) stained sections from WT, VDRKO and 1αKO glands at 9 and 16 days pregnancy (Timed Mating) and 3 and 6 days involution illustrates the growth of epithelial tissue during pregnancy and the regression of epithelial cell structures during involution. The data shows that the VDRKO glands possess more epithelial tissue during 9 and 16 days pregnancy (p-values 0.044 and 0.036) and 3 days involution (p=0.002) There is no difference in the amount of epithelial tissue in the 1αKO mammary glands compared to the WT glands, suggesting similar control of epithelial cell growth and regression in these glands. (asterisks indicate statistical difference to the WT group at that time point calculated by student's t-test).

(Subtask 7) Assessment of proliferation via BrdU staining of paraffin embedded sections (months 8-10) has yet to be completed. We have restricted this analysis to the animals at 9 and 16 days of pregnancy as this is the most important phase during the pregnancy cycle in terms of proliferation. We have had some issues with our BrdU antibody and have obtained a replacement. If this fails, we will use a Ki-67 antibody as a marker of proliferation. This will be completed along with proliferation analysis of our organ culture studies before month 30. We have previously reported that the initiation of proliferation during the first half of pregnancy has been seen to be faster in VDRKO mice than in the wild type controls. (Subtask 8) Assessment of apoptosis using TUNEL staining of sections (months 8-10) has been completed. We looked specifically at apoptosis in involution samples, as that is when apoptosis is most relevant. A protocol change has been implemented for the BrdU and TUNEL staining of sections. We believe we can achieve a more accurate quantification of positive cells with a fluorescent-tagged antibody rather than the colorimetric approach. We have collected the data for the TUNEL assay quantification and is illustrated in figure 6. A 2-fold increase in apoptotic signaling in our two knockout models compared to the wild type controls is demonstrated at 3 days involution. This is not observed at 6 days involution as no statistical difference is shown.

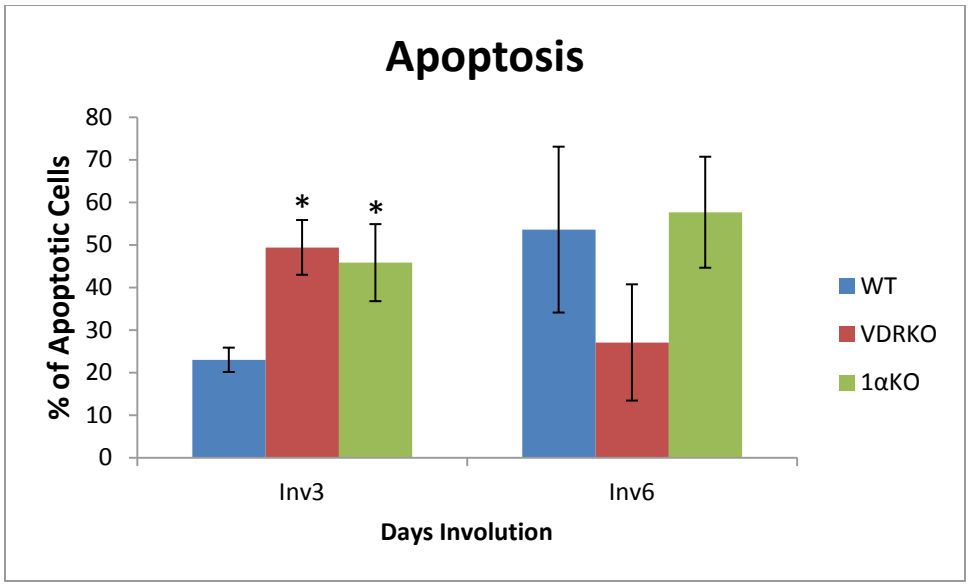

Figure 6. Quantification of fluorescent TUNEL-stained sections from WT, VDRKO and 1αKO glands at 3 and 6 days involution illustrates the regression of epithelial cell structures during involution as a measure of apoptosis. The data shows that there is an increased amount of apoptosis in the VDRKO and 1αKO glands during the 3rd day of involution (p-values 0.005 and 0.003), suggesting there are similarities in apoptotic signaling in these glands. (asterisks indicate statistical difference to the WT group at that time point calculated by student's t-test).

(Subtask 9) Isolation of RNA from thoracic mammary glands (months 10-12) has been completed and qPCR for each stage of pregnancy was performed. An increase in VDR expression was observed in the 1αKO glands on the 9th day of pregnancy. (Subtask 10) This coincided with an increase of p21 and cyclin D1 expression (figure 7). In glands lacking the VDR, an observed increase in cyclin D1 mRNA and wild type levels of p21, demonstrates the complex signaling of the vitamin D pathway. At 16 days pregnancy, VDR expression increases 2-3 fold compared to levels at 9 days pregnancy but both p21 and cyclin D1 levels decrease and result in similar expression in all three models. We intend to analyze additional genes to try to explain these results. At 5 days lactation, VDR levels in 1αKO mammary glands are elevated, as was the marker of differentiation TGF-β but not β-casein (figure 8). TGF-β expression is also elevated in VDRKO glands but β-casein is not statistically increased. 5 days later, VDR and TGF-β expression is again increased in 1αKO samples compared to their wild type counterparts, this time with the addition of β-casein (figure 9). To analyze apoptotic signaling during involution, we chose to observe pro-apoptotic bax and anti-apoptotic bcl2 expression levels. At 3 days involution, none of the genes measured in our 2 knockout models (VDR, cyp24, bax, bcl2) had levels significantly different than that of the wild type glands other than the obvious lack of VDR in the VDRKO group. Interestingly, in the 1αKO glands, both bax and bcl2 levels are elevated at 6 days involution as well as cyp24 (figure 10) compared to that of the wild type controls.

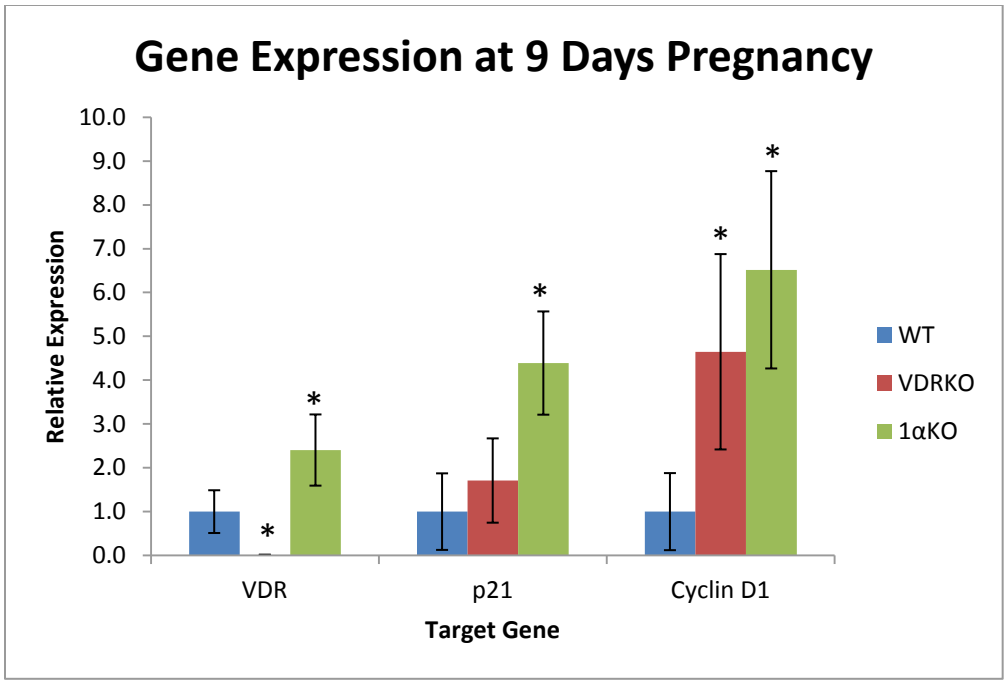

Figure 7. Gene expression analysis of WT, VDRKO and 1αKO glands at 9 days pregnancy shows an increase of VDR levels in the 1αKO glands (p=0.004) which coincides with increased expression of p21 (p=0.0002) and cyclin D1 mRNA (p=0.0001). Glands lacking the VDR still demonstrate an increase in cyclin D1 expression at this time (p=0.003) but lack an increase in p21 shown in the 1αKO glands. (asterisks indicate statistical difference to the WT group for that gene calculated by student's t-test)

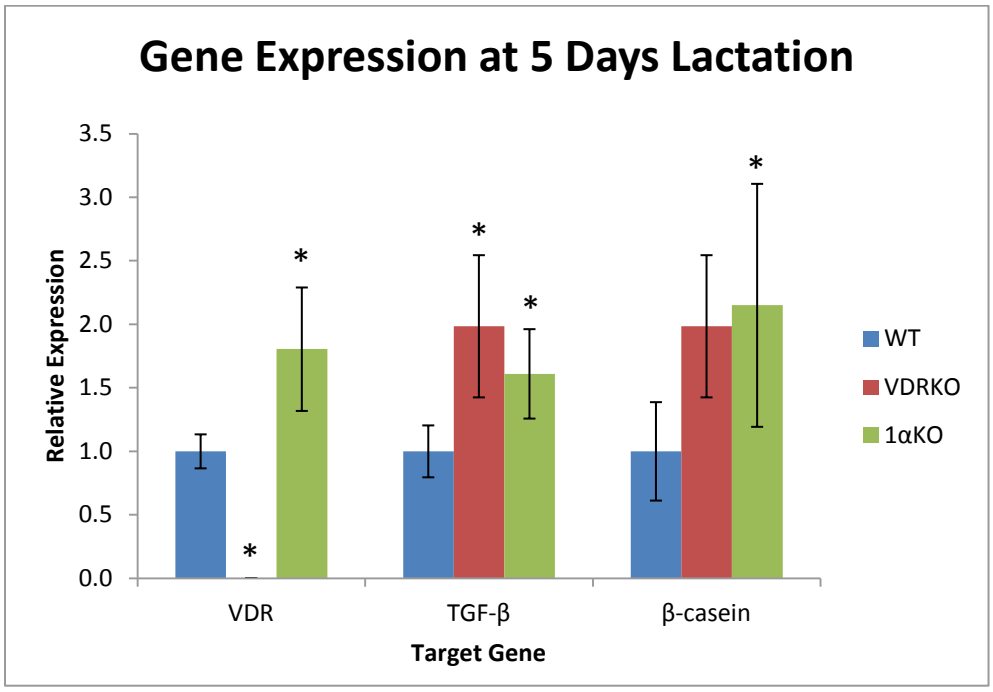

Figure 8. Gene expression analysis of WT, VDRKO and 1αKO glands at 5 days lactation shows an increase of VDR levels in the 1αKO glands (p=0.007) which coincides with increased expression of TGF-β mRNA (p=0.010). Glands lacking the VDR still demonstrate an increase in TGF-β (p=0.010). An increase in β-casein levels was found in 1αKO glands (p=0.038). (asterisks indicate statistical difference to the WT group for that gene calculated by student's t-test)

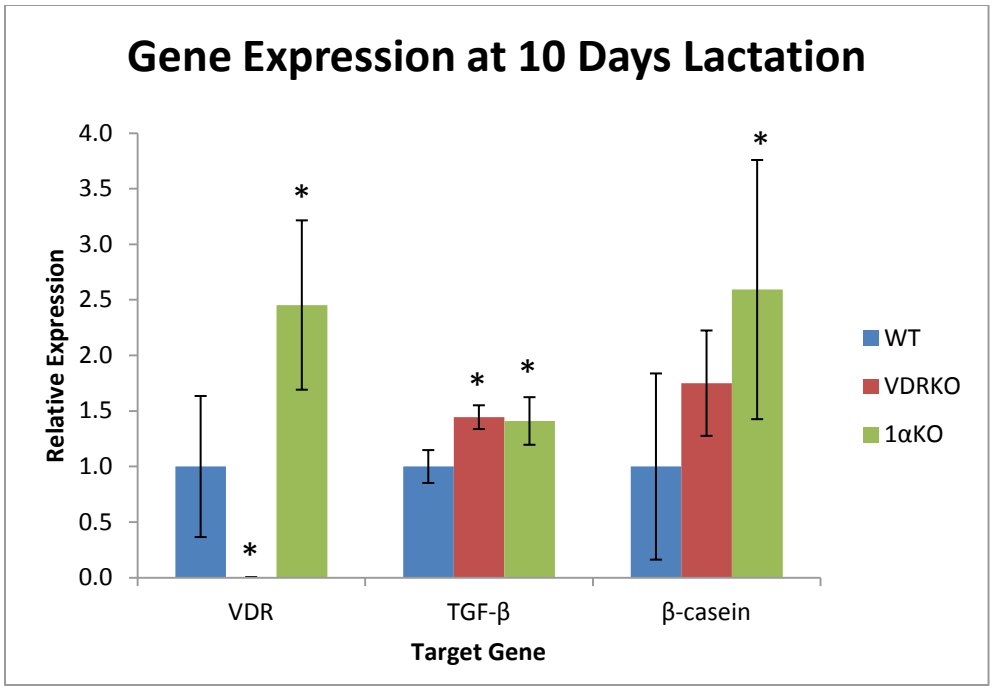

Figure 9. Gene expression analysis of WT, VDRKO and 1αKO glands at 10 days lactation shows an increase of VDR levels in the 1αKO glands (p=0.008) which coincides with increased expression of TGF-β (p=0.006) and β –casein mRNA (p=0.031). Glands lacking the VDR show an increase in TGF- β mRNA (p=0.013) but no statistical difference in β-casein levels. (asterisks indicate statistical difference to the WT group for that gene calculated by student's t-test)

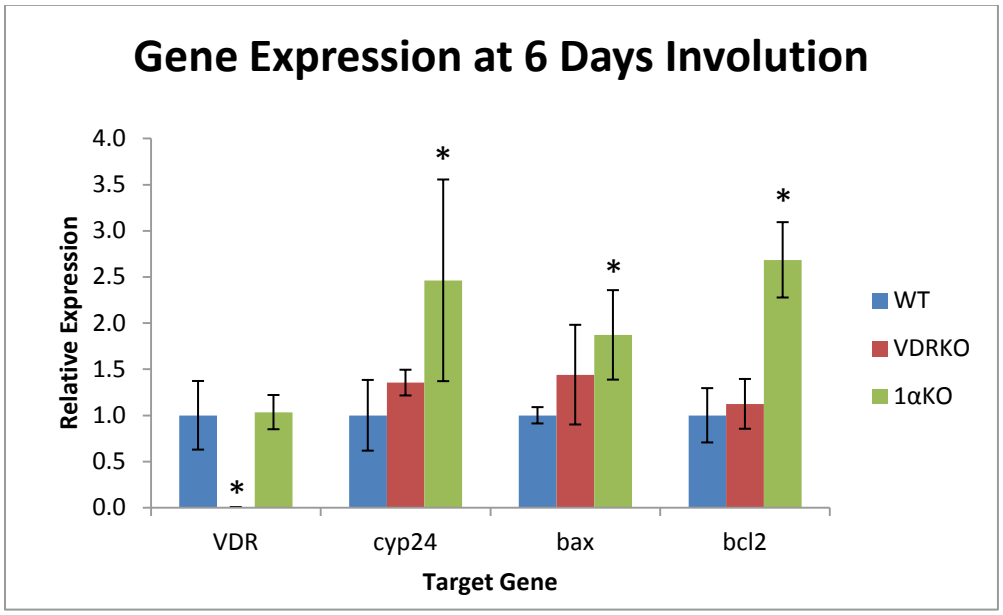

Figure 10. Gene expression analysis of WT, VDRKO and 1αKO glands at 6 days involution shows no increase of VDR levels in the 1αKO glands but has an increase in the vitamin D pathway self-regulating cyp24 gene mRNA (p=0.032). 1αKO glands also have elevated levels of pro and anti-apoptotic genes, bax (p=0.007) and bcl2 (p<0.001). Glands lacking the VDR show no statistical difference in cyp24, bax or bcl2 levels. (asterisks indicate statistical difference to the WT group for that gene calculated by student's t-test)

(Subtask 11) Isolation of protein from thoracic mammary glands has been completed (month 12). (Subtask 12) Western blot analysis is still ongoing and should be completed by month 30. We will be looking at the same genes as we are analyzing with qPCR and will be completing this in parallel with our organ culture studies.

(Subtask 13) Annual results were presented in our departmental seminar. (Subtask 14) The manuscript for *in vivo* experiments is still in progress.

***Ex vivo* analysis of mammary gland development and treatment with vitamin D metabolites**

(Subtask 1) During the delay in the initial setup of the mouse pairings for task 1, we optimized the organ culture conditions (month 13) earlier than expected. (Subtask 2) We maintained hormone pellet implantation for 2 weeks and cultured the glands for 7 days (figure 11) (Subtask 3) while treating with 100nM 1,25-dihydroxyvitamin D, 500nM 25-hydroxyvitamin D or ethanol control.

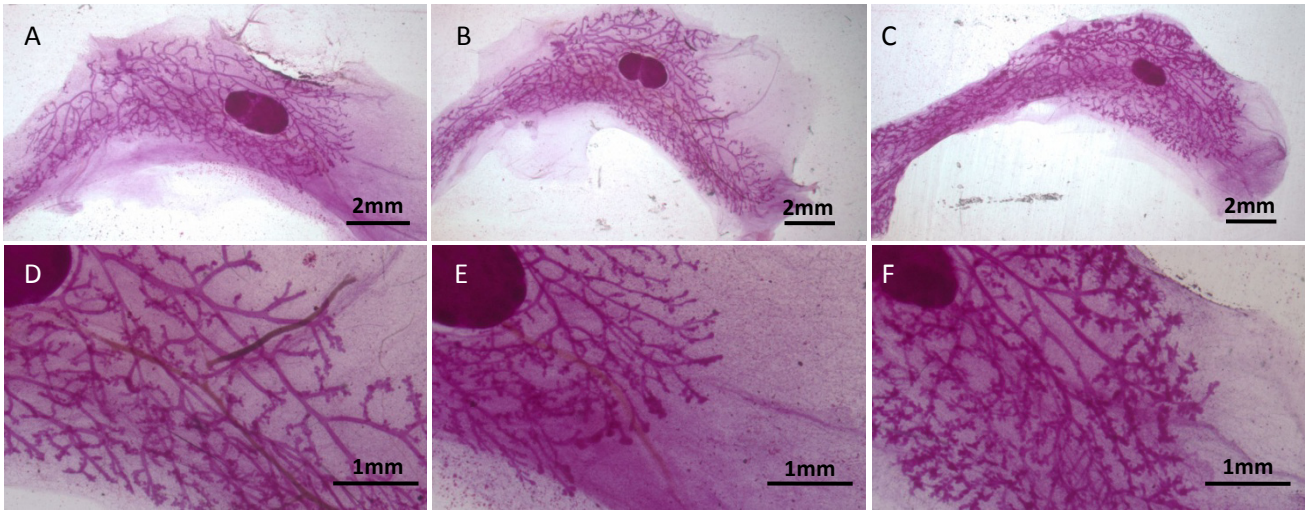

Figure 11. Whole mounts of organ cultured mammary glands illustrating the effects of hormone supplementation *ex vivo*. Wild type mice were used ((A,D) control at day 0) and glands were grown in culture for (B,E) 1 day and (C,F) 7 days in media containing a hormone cocktail. Top pictures were taken at 3.5x magnification. Bottom pictures were taken at 16x magnification.

(Subtask 4) Whole mount observations (months 15-30) of *ex vivo* mammary glands concluded that treatment of wild type and 1αKO glands with 1,25D impeded the ability of the ductal branching network to form a large proportion of their secondary and tertiary branches (figure 12). Treatment with 100nM 1,25D did not have a dramatic impact on VDRKO mammary gland branching. Also, treatment with 500nM 25D did not appear to have an effect on the branching of any of the three models. (Subtask 5) Ductal outgrowth analysis demonstrated the impact of 1,25D on the different genotypes, resulting in the significantly reduced branching extension of the wild type and 1αKO ductal networks compared to the VDRKO (figure 13). In addition, it was determined that the lymph nodes within the inguinal mammary glands of VDRKO mice are larger than those of wild type or 1αKO glands (figure 14). (Subtask 6) Terminal end bud formation quantitative analysis does not appear to be feasible with the presently implemented technology. The use of ImageJ software to elicit any changes in epithelial tissue area

within parts of the whole mounted glands is proving to be difficult even with the use of dynamic thresholding. Other techniques to more specifically quantify tertiary branching instead of overall epithelial cell density are currently being explored. (months 16-31).

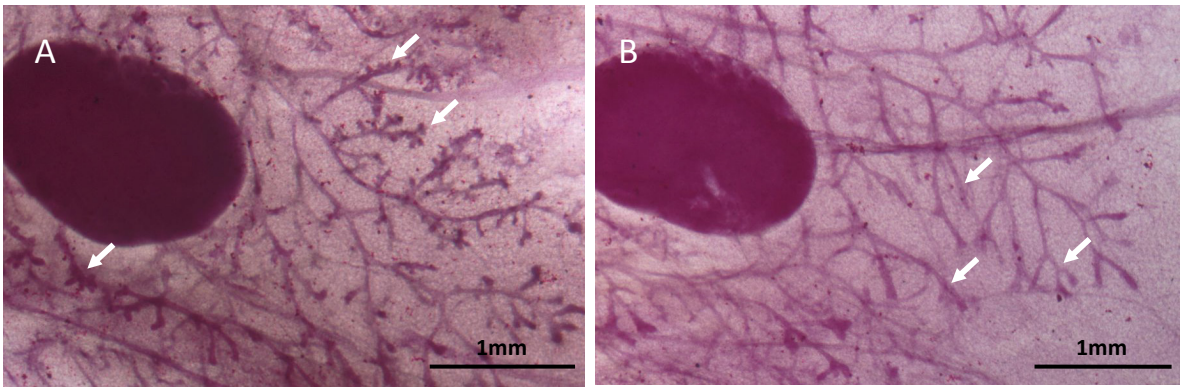

Figure 12. Mammary glands were treated in culture with 500nM 25D, 100nM 1,25D or ethanol control. Shown are wild type glands treated with (A) ethanol and (B) 1,25D. Treatment with the vitamin D metabolite prevented the secondary and tertiary branching structures (arrows) that would normally form upon signaling from the hormone cocktail in culture.

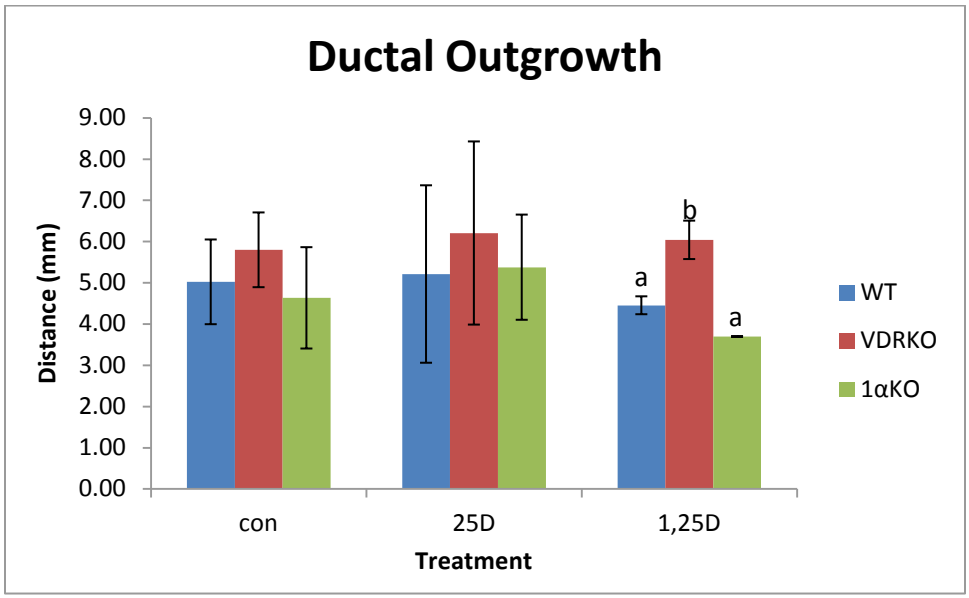

Figure 13. Ductal outgrowth analysis of WT, VDRKO and 1αKO mammary glands grown in culture and treated with 500nM 25D, 100nM 1,25D or ethanol control. Treatment of glands with the active metabolite of vitamin D, 1,25D elicited a difference in the extension of the ductal branching network into the mammary fat pad between the VDRKO model and the 1αKO and WT models (p=0.001). (letters indicate statistical difference to the WT group for that treatment calculated by ANOVA)

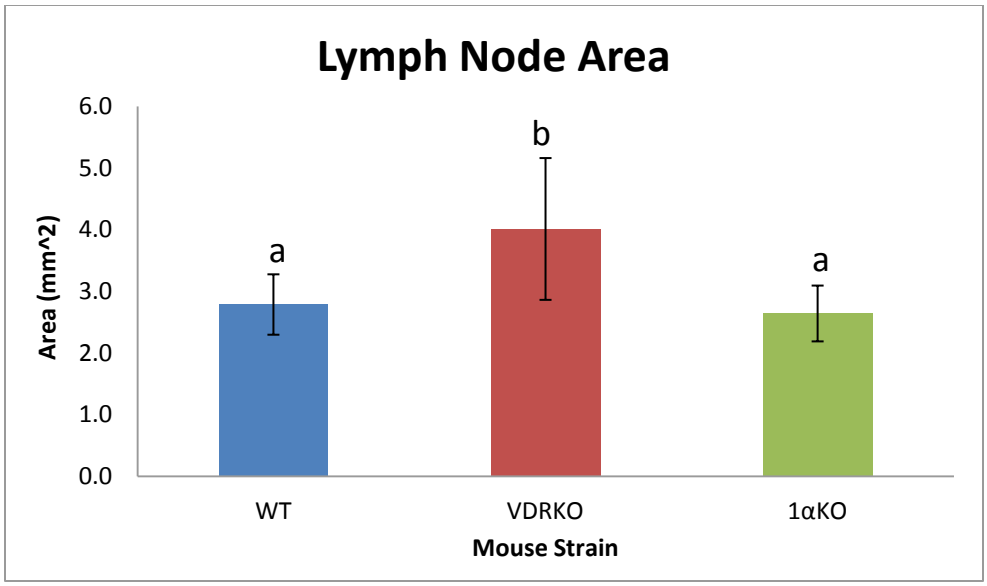

Figure 14. Measurements of the lymph node area of WT, VDRKO and 1αKO mammary glands grown in culture. Lymph nodes present in the inguinal glands of VDRKO mice are consistently larger than those from WT or 1αKO glands (p=0.0003). (letters indicate statistical difference to the WT group calculated by ANOVA)

(Subtask 7) Inguinal glands were formalin fixed, dehydrated and embedded in paraffin wax (months 15-30). These blocks were then sectioned and H&E stained. Analysis of H&E stained sections (months 16-31) to determine the amount of epithelial cells present in *ex vivo* glands demonstrated no differences between strains. Treatment of wild type glands with 25D shows a decrease in epithelial tissue area but we believe this to be an artifact of the tissue sectioning process (figure 15). (Subtask 8) Preliminary observations suggest there is no statistical difference in presence of epithelial cells due to treatment. Additional sections have been set aside for immunohistochemical analysis at a future date (months 16-42). (Subtask 9) Ki67 presence will be measured to determine the level of proliferation in each of our models and treatments. (Subtask 10) TUNEL assays will also be performed to elucidate the presence of apoptosis and any changes that result from vitamin D treatment (months 18-42).

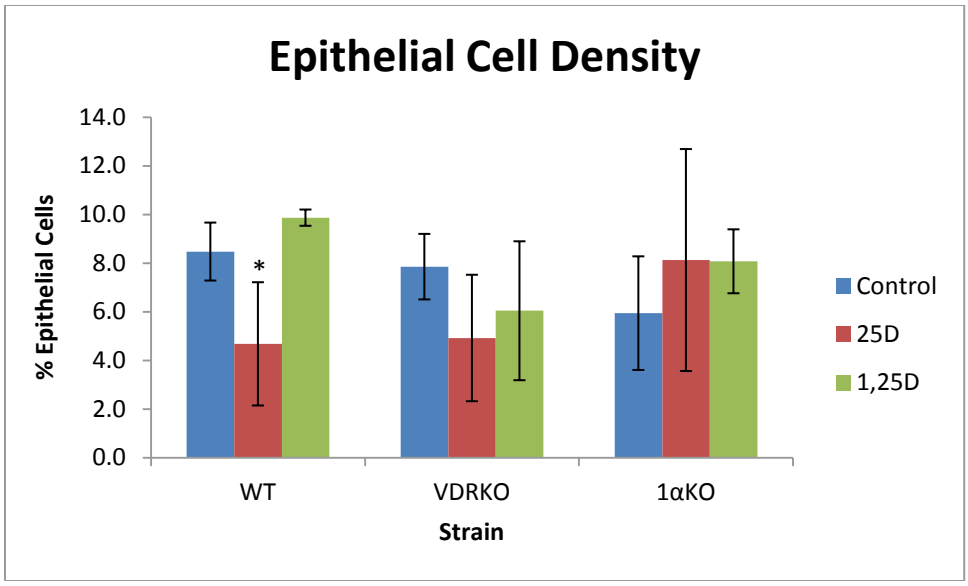

Figure 15. Analysis of epithelial cell area in H&E stained mammary gland sections from each strain of mouse and treated with either 25D or 1,25D (or ethanol control). A statistically significant decrease was observed in the wild type mice with 25D treatment (p=0.008) but is likely due to sections not being in the same plane as the rest of the samples. (asterisks indicate statistical difference to the WT control group calculated by ANOVA)

(Subtasks 11-12) We have isolated RNA from all of our thoracic gland samples and have used qPCR to analyze a number of genes. No differences were found in a number of different genes analyzed, including cyclinD1, Bax, Bcl2 and Cyp24. Basal levels of p21 were found to be slightly elevated in control KO glands (VDKRO and 1αKO) but were unaffected by treatment with either 25D or 1,25D (figure 16). Further gene expression analysis will be performed for additional targets. (months 16-31). (Subtasks 13-14) Microarray analysis showed changes in gene expression upon treatment of WT glands with 1,25D in culture. The genes of interest are listed in Table 1. Protein was harvested from these thoracic glands (months 30-42) and western blot analysis will be performed to verify mRNA expression changes with those at the protein level as per the revised statement of work (months 30-42).

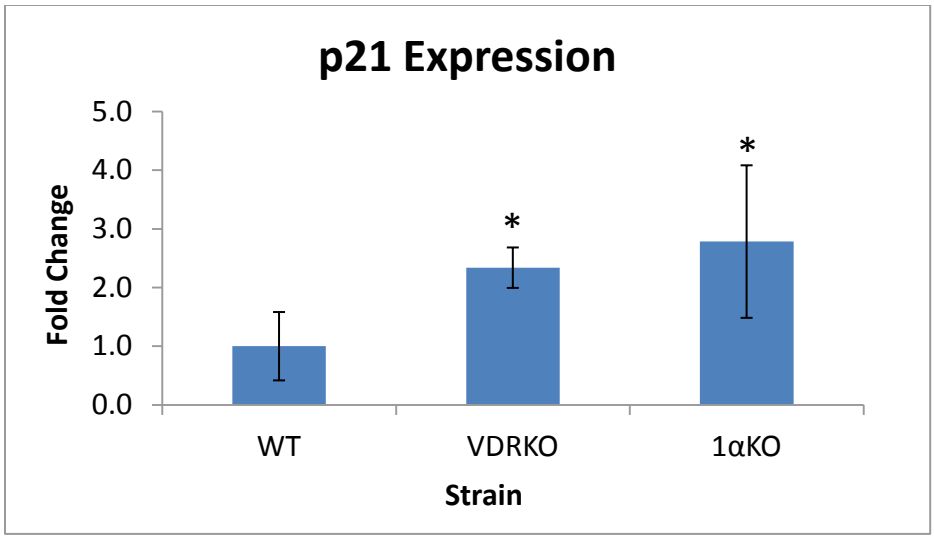

Figure 16. Gene expression analysis of WT, VDRKO and 1αKO glands in organ culture shows a basal increase of p21 levels in the VDRKO and 1αKO glands (p=0.002 and p=0.023 respectively). (asterisks indicate statistical difference to the WT group for that gene calculated by student's t-test)

Table 1. Mouse exon expression analysis of WT 1,25D treated vs control mammary glands in organ culture. Listed are the statistically significant changes in expression of both canonical calcium regulatory genes and other possible genes of interest. (Fold change cutoff was set at 1.5)

Calcium Regulatory Genes

Fold Change	Gene Symbol	Gene description
1.6	**Camk2a**	calcium/calmodulin-dependent protein kinase II alpha
-1.51	**Clca4**	chloride channel calcium activated 4
-1.52	**S100a4**	S100 calcium binding protein A4
-1.55	**Camta1**	calmodulin binding transcription activator 1
-1.62	**Calca**	calcitonin/calcitonin-related polypeptide, alpha
-1.62	**Casq2**	calsequestrin 2
-1.88	**Calml3**	calmodulin-like 3

Other Genes of Interest

Fold Change	Gene Symbol	Gene Description
1.72	**Cyp24a1**	cytochrome P450, family 24, subfamily a, polypeptide 1
1.63	**Cited1**	Cbp/p300-interacting transactivator w/ Glu/Asp-rich carboxy-terminal domain 1
1.56	**Steap2**	six transmembrane epithelial antigen of prostate 2
1.5	**Lpin3**	lipin 3
-2.61	**Csn1s2b**	casein alpha s2-like B

(Subtask 15) I will be presenting my new results in addition to the results from the ongoing organ culture studies in February 2014. (Subtask 16) Upon completion of each task, a manuscript will be prepared and submitted for publication.

Key Research Accomplishments

Task 1

- An increase in ductal growth was observed in VDRKO glands during puberty, compared to WT and 1αKO glands
- An increase in ductal branching was observed in VDRKO glands during pregnancy, compared to WT and 1αKO glands
- A delay in involution at 3 days is observed in VDRKO and 1αKO glands, demonstrated by persistent alveoli compared to WT glands
- Invasion of mammary gland tissue by stromal cells at 3 days involution appears more extensive in VDRKO glands compared to WT and 1αKO glands
- An increase in apoptotic cells at 3 days involution is observed in VDRKO and 1αKO glands compared to WT glands
- VDR mRNA is elevated in all 1αKO glands during all stages except involution compared to VDRKO and WT glands
- Increased Bax and Bcl2 mRNA is elevated in 1αKO glands at 6 days involution compared to WT glands. VDRKO may have increased Bax levels at this time point (as described previously) but sample size may be too small.
- An increase in cyclin D1 levels at 9 days pregnancy is observed in VDRKO and 1αKO glands compared to WT glands. Interestingly, p21 levels in 1αKO glands are also elevated during this time point compared to WT and VDRKO glands.
- An increase in TFG-β at 5 days lactation is observed in VDRKO and 1αKO glands compared to WT glands. An increase in β-casein is also observed in 1αKO glands at 5 and 10 days lactation compared to WT glands

Task 2

- Treatment of WT and 1αKO glands with 1,25D in culture decreases ductal outgrowth and branching compared to VDRKO glands
- There was no effect on ductal morphology in any strain upon treatment with 25D
- It was observed that VDRKO glands possess larger lymph nodes than that of WT or 1αKO glands
- There were no changes in epithelial tissue area within the glands of any strain in culture upon treatment with 25D or 1,25D
- Microarray analysis showed that treatment of WT glands with 1,25D induces changes in both calcium regulatory genes and non-canonical vitamin D pathway genes.
- qPCR analysis demonstrated that KO glands both show a basal increase in p21 levels compared to WT glands.

Reportable Outcomes

Matthews DG et al. Vitamin D pathway status and the identification of target genes in the mouse mammary gland. AACR Special Conference: Advances in Breast Cancer Research. San Francisco, CA, Oct 12-15, 2011.

Matthews DG et al. Characterization of mammary gland development in vitamin D pathway ablated mice. 15th Vitamin D meeting. Houston Texas, June 20-22, 2012.

Conclusion

All samples for task one were collected. VDR levels were consistently higher throughout pregnancy and lactation in 1αKO glands, perhaps as a mechanism to attempt to overcome a lack of available active vitamin D. Analysis of whole mounts and H&E stained sections of 9 and 16 day pregnancy samples verify an increase in the presence of epithelial cells due to accelerated ductal branching in VDRKO glands compared to wild type and 1αKO glands. This correlates with elevated cyclin D1 mRNA levels and suggests that the VDR acts independently of 1,25 dihydroxyvitamin D to mediate these results. This data will be verified with the completion of western blot protein analysis. Interestingly, 1αKO glands display elevated levels of both pro and anti-proliferative genes cyclin D1 and p21 at 9 days pregnancy, something not observed in the other two models. This may be due to an alternate function of the 1α-hydroxylase enzyme, adding another layer of complexity to vitamin D pathway studies. During lactation, we observed an increase in TGF-β signaling in both knockout models which indicates a stronger push towards differentiation in these glands and also illustrates some similarity between our vitamin D pathway aberrant models. Involution samples sectioned and H&E stained illustrate a delayed apoptotic response in the 1αKO glands, similar to what was previously observed in VDRKO glands in terms of alveolar deconstruction 3 days into involution in some animals. However, we do observe an elevated level of apoptosis in both knockout models at this time point, which does not appear to be the result of bax/bcl2 regulation, possibly due to a lag in apoptotic signaling in these glands. Organ culture studies have confirmed a more extensive branching network in VDRKO glands treated with 1,25D compared to the wild type and 1αKO models, indicating that 1αKO glands are able to utilize vitamin D metabolites downstream of the ablated activating enzyme. Whole mount analysis has also demonstrated that treatment of glands with 1,25D will prevent a significant amount of the secondary and tertiary branching observed in the control glands under hormonal stimulation. Even though these observed effects are evident in the mammary gland as a whole, differences in the quantification of gene expression changes via microarray and qPCR analysis appear to be minimal at this time. Perhaps other putative target genes or alterations in experimental methods could be used to enhance the elucidation of these changes upon treatment and are currently being explored. In addition, protein analysis using western blotting and immunohistochemical techniques may yet yield more definitive results. The completion of these studies will give us a better idea of how vitamin D affects epithelial cell growth in the mammary gland and provide insight into the correlation between vitamin D deficiency and an increase in breast cancer incidence.

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APPENDIX A

Copy of Abstract for AACR Advances in Breast Cancer Research Meeting (Oct 12-15, 2011)

Vitamin D pathway status and the identification of target genes in the mouse mammary gland

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Multiple epidemiological studies have demonstrated correlations between vitamin D status and incidence of cancers, including colon and breast. Our long term goal is to understand the mechanisms by which the vitamin D signaling pathway impacts on breast cancer. Upon absorption or endogenous synthesis, vitamin D is metabolized into 25-hydroxyvitamin D (25D) and 1,25-dihydroxyvitamin D (1,25D) in multiple tissues. 1,25D is a high-affinity ligand for the nuclear vitamin D receptor (VDR) that alters gene expression and inhibits growth of normal and transformed mammary cells. However, 25D and other steroid-like compounds such as bile acids, can also activate VDR *in vitro*. Although studies with VDR knockout (KO) mice have demonstrated that VDR affects proliferation, differentiation and apoptosis in the mammary gland, the specific ligands that trigger VDR signaling have yet to be identified. Generation of 1,25D from 25D is mediated by CYP27B1 which is present and developmentally regulated in the mammary gland. Studies with the VDRKO mouse have established that its phenotype is different from mice lacking CYP27B1, supporting the concept that other VDR ligands exist and/or the VDR may act in the absence of ligand. We are using animal models to determine whether VDR exerts 1,25D-independent effects on growth, apoptosis or differentiation of mammary epithelial cells *in vivo*. Glands from VDRKO and CYP27B1KO mice during puberty, pregnancy, lactation, involution and aging have been collected for analysis of gross morphology, histology, and genomic profiling in comparison to age-matched control mice. Our data indicate that mammary glands from VDRKO mice display accelerated branching, enhanced sensitivity to estrogen and progesterone and altered gene expression during puberty and early pregnancy. Surprisingly, initial studies indicate that similar changes are not observed in glands from CYP27B1KO mice, suggesting that other VDR ligands may substitute for 1,25D in control of mammary cell turnover *in vivo*. Alternatively, our results are consistent with the concept that unoccupied VDR may function in the mammary gland. To further explore the role of VDR and CYP27B1 in the glandular epithelial compartment, mice with mammary epithelial-specific deletion of VDR or CYP27B1 were generated by crossing MMTV- cre mice with mice carrying floxed alleles of VDR or CYP27B1. Quantitation of VDR and CYP27B1 expression in the mammary gland, and characterization of the phenotype of these cre-flox mice is in progress. Collectively, these studies have identified novel roles for the VDR and its ligands in mammary gland development that provide insight into the relationship between vitamin D status and breast cancer.

Copy of Abstract for 15th Vitamin D Workshop (June 20-22, 2012)

CHARACTERIZATION OF MAMMARY GLAND DEVELOPMENT IN VITAMIN D PATHWAY ABLATED MICE.

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Multiple epidemiological studies have demonstrated correlations between vitamin D status and incidence of cancers, including colon and breast. Our long term goal is to understand the mechanisms by which the vitamin D signaling pathway impacts on breast cancer. Generation of the high-affinity vitamin D receptor (VDR) ligand 1,25-dihydroxyvitamin D (1,25D) from 25-hydroxyvitamin D (25D) is mediated by CYP27B1 which is present and developmentally regulated in multiple tissues including the mammary gland. Studies with the VDR knockout (VDRKO) mouse have established that its phenotype is different from mice lacking CYP27B1, supporting the concept that other VDR ligands exist and/or the VDR may act in the absence of ligand. We are using animal models to determine whether VDR exerts 1,25D-independent effects on growth, apoptosis or differentiation of mammary epithelial cells *in vivo*. Glands from VDRKO and CYP27B1KO mice during puberty, pregnancy, lactation, involution and aging have been collected for analysis of gross morphology, histology, and genomic profiling in comparison to age-matched control mice. Our data indicate that mammary glands from VDRKO mice display accelerated branching, enhanced sensitivity to estrogen and progesterone and altered gene expression during puberty and early pregnancy. Surprisingly, initial studies indicate that similar changes are not observed in glands from CYP27B1KO mice although gene expression changes associated with cell cycle regulation (p21, cyclin D1), differentiation (TGF-β, β-casein) and apoptosis (Bax) have been noted. This suggests that other VDR ligands may substitute for 1,25D in control of mammary cell turnover *in vivo*. Alternatively, our results are consistent with the concept that unoccupied VDR may function in the mammary gland. Collectively, these studies have identified novel roles for the VDR and its ligands in mammary gland development that provide insight into the relationship between vitamin D status and breast cancer. Funded by DOD Predoctoral Fellowship Award #W81XWH-11-10152

Annual Training Summary

2011

April 6, 2011 – Topics in Cancer, Journal Club Presentation – Gave 1hr presentation on relevant and current cancer research. Paper presented – Trimboli AJ et al. *Pten* in Stromal Fibroblasts Suppresses Mammary Epithelial Tumors. Nature 2009;461(7267):1084-91.

October 13, 2011 – Poster Presentation – Presented current data to interested parties for 2hrs. Matthews DG et al. Vitamin D pathway status and the identification of target genes in the mouse mammary gland. AACR Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications meeting, San Francisco California.

November 2, 2011 – Topics in Cancer, Journal Club Presentation – Gave 1hr presentation on relevant and current cancer research. Paper presented – Lyons TR et al. Postpartum mammary gland involution drives progression of ductal carcinoma *in situ* through collagen and COX-2. Nature Medicine 2011;17(9):1109-15.

November 5, 2011 – Annual Departmental Seminar – Gave 1hr presentation on current research progress. Matthews DG. Vitamin D pathway status and the identification of target genes in the mouse mammary gland.

2012

March 27, 2012 – Topics in Cancer, Journal Club Presentation – Gave 1hr presentation on relevant and current cancer research. Paper presented - Png KJ et al. A microRNA regulon that mediates endothelial recruitment and metastasis by cancer cells. Nature 2011;481(7380):190-4.

May 18, 2012 – Annual Departmental Seminar – Gave 1hr presentation on current research progress. Matthews DG. Vitamin D pathway status and the identification of target genes in the mouse mammary gland.

October 3, 2012 – Topics in Cancer, Journal Club Presentation – Gave 1hr presentation on relevant and current cancer research. Paper presented - Li J et al. Treatment of breast cancer stem cells with oncolytic herpes simplex virus. Cancer Gene Therapy 2012;19(10):707-14.

June 21, 2012 – Poster Presentation – Presented current data to interested parties for 2hrs. Matthews DG et al. Vitamin D pathway status and the identification of target genes in the mouse mammary gland. 15th Workshop on Vitamin D, Houston Texas.

2013

February 27, 2013 – Topics in Cancer, Journal Club Presentation – Gave 1hr presentation on relevant and current cancer research. Paper presented – Yin G et al. Constitutive proteasomal degradation of TWIST-1 in epithelial-ovarian cancer stem cells impacts differentiation and metastatic potential. Oncogene 2013; 32, 39-49.

October 16, 2013 - Topics in Cancer, Journal Club Presentation – Gave 1hr presentation on relevant and current cancer research. Paper presented – Werbeck JL et al. Tumor Microenvironment Regulates Metastasis and Metastasis Genes of Mouse MMTV-PymT Mammary Cancer Cells In Vivo. Veterinary Pathology 2013 Oct 3, Epub.